Overexpression of a *Rhizopus delemar* Lipase Gene in *Escherichia coli*[1]

A cloned complementary deoxyribonucleic acid encoding the precursor polypeptide of an extracellular lipase from the fungus *Rhizopus delemar* was altered by site-directed mutagenesis to generate deoxyribonucleic acid fragments that specifically code for the polypeptides of the proenzyme and the mature form of the lipase. Attempts to produce these polypeptides in enzymatically active form in *Escherichia coli* revealed toxic effects toward the host. Therefore the polypeptides were expressed as inactive and insoluble forms in the cytoplasm of *E. coli* BL21 (DE3) cells using plasmid vector pET11-d. With this tightly regulated high-level expression system, lipase and prolipase polypeptides were produced to estimated levels of up to 21% and 15%, respectively, of total cellular protein. The insoluble polypeptides were solubilized in 8 M urea. Refolding into active forms was achieved by treatment with the redox system cystine/cysteine and dilution. Refolded mature lipase was purified to homogeneity by affinity and ion exchange chromatography. The enzyme had a specific activity comparable to that of lipase from the fungal culture. The quantities of pure enzyme obtained from a 1-L culture of *E. coli* exceeded those obtained from the fungal culture by a factor of at least 100. Refolded recombinant prolipase was purified essentially to homogeneity and had a specific activity similar to that of the mature enzyme. Its pH optimum was 7.5, rather than the pH 8 determined for recombinant mature lipase and for the enzyme purified from the fungal culture. Recombinant prolipase retained activity after 15 min incubation at 65°C, while mature lipase retained activity only up to 45°C.
Lipids 28, 81–88 (1993).

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3) are enzymes capable of hydrolyzing ester bonds of water-insoluble substrates at the substrate-water interface. Lipolytic enzymes are ubiquitous and their physical and biochemical diversity provides the basis for many current and future biotechnological uses. In nature, the hydrolysis of acylglycerols is probably the main function of lipases. Under laboratory conditions, lipases are able to catalyze transesterification and ester synthesis reactions with alcohols other than glycerol and to hydrolyze a diversity of esters in nonaqueous solvent systems (1).

The fungus *Rhizopus delemar* (presently designated *R. oryzae*) produces extracellular lipases that hydrolyze the ester bonds at the *sn*-1 and *sn*-3, but not the *sn*-2 position, of a triacylglycerol (2). This pronounced positional specificity is one of the reasons for continued basic studies on the *R. delemar* lipases and for the use of partially purified *R. delemar* enzyme preparations in acylglycerol restructuring (3), the exchange of acyl groups of phospholipids (4) and ester and glyceride synthesis (5–7).

Industrial applications of lipases and basic scientific experiments are often hampered by the lack of sufficient enzyme or by the heterogeneity of the available preparations. The complete purification of the *R. delemar* lipase is time-consuming and yields only about 0.2–0.6 mg per liter of culture supernatant (8). Low purification yields have prompted researchers to employ molecular cloning techniques to improve the production of lipases. For example, the complementary DNA (cDNAs) for extracellular lipases from *Rhizomucor miehei* and *Humicola lanuginosa* were overexpressed in the fungus *Aspergillus oryzae*, and mature lipase was purified from the culture medium (9,10). The gene for a thermostable lipase from the bacterium *Pseudomonas fluorescens* was overexpressed in *Escherichia coli* to a level of 40% of the total protein (11).

The use of recombinant DNA techniques for the production of an *R. delemar* lipase became possible after a cDNA clone of the lipase was obtained and its nucleotide sequence was determined (12). The sequence data predict that the fungus produces a mRNA that is translated into a preproenzyme. The export signal peptide and the propeptide are then removed proteolytically to produce the mature lipase enzyme. Here we report on the cloning of DNAs encoding the prolipase and the mature lipase in *E. coli*, their expression in the bacterium in inactive form, their refolding *in vitro* to active lipolytic proteins, and their purification.

MATERIALS AND METHODS

DNA manipulations. Plasmid DNA and phage M13 RF DNA were isolated by an alkaline lysis procedure (13) followed by a phenol/chloroform extraction. Restriction enzyme digestions and ligation reactions were performed under conditions recommended by the suppliers. Transformation with plasmid DNA was accomplished using $CaCl_2$-treated *E. coli* cells as described by Sambrook *et al.* (14).

Construction of lipase expression plasmids. The previously cloned cDNA encoding the *R. delemar* lipase (12) was excised from plasmid pUC8-2.14 (12) as an *Eco*RI fragment and inserted into the *Eco*RI site of bacteriophage M13 mp18 (15). Site-directed mutagenesis of the insert DNA was carried out according to the method of Kunkel (16) using the MUTA-GENE M13 *IN VITRO* MUTAGENESIS KIT (Bio-Rad Laboratories, Richmond, CA). Mutagenic primers were obtained from the Department of Chemistry, University of Pennsylvania, Philadelphia, PA. A primer, 5′GGTTCATCCATGGTTCCTGT 3′, was used to replace the codon for the C-terminal amino acid, alanine, of the predicted signal peptide of preprolipase with a codon representing methionine. This primer also changes the nucleotide sequence to introduce an *Nco*I

[1]Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: bp, base pairs; cDNA, compementary deoxyribonucleic acid; CM-Sepharose, carboxymethyl Sepharose; DNA, deoxyribonucleic acid; DTT, diethiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; OA, oleic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; U, units of lipase activity.

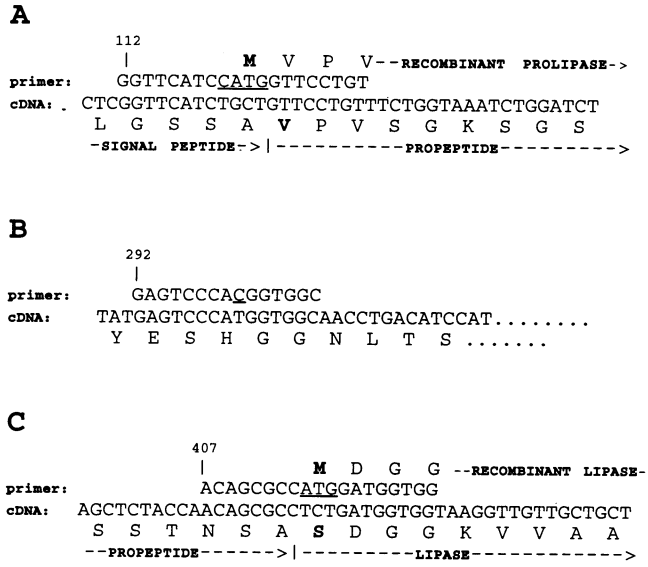

FIG. 1. Oligonucleotide primers used for mutagensis of the *R. delemar* lipase cDNA. A. Primer for the introduction of a methionine codon and an *Nco*I site at the position in the *R. delemar* lipase cDNA encoding the junction between signal peptide and propeptide. B. Primer for the removal of an *Nco*I site within the propeptide encoding region. C. Primer for the introduction of a methionine codon and an *Nco*I site at the position in the lipase cDNA encoding the junction between propetide and mature lipase. The amino acid sequence predicted from the cDNA is given below the nucleotide sequence. The amino acid sequence resulting from the oligonucleotide directed mutagenesis is given above the primer sequence. The numbers indicate positions of nucleotides in the published nucleotide sequence of the *R. delemar* cDNA (ref. 12).

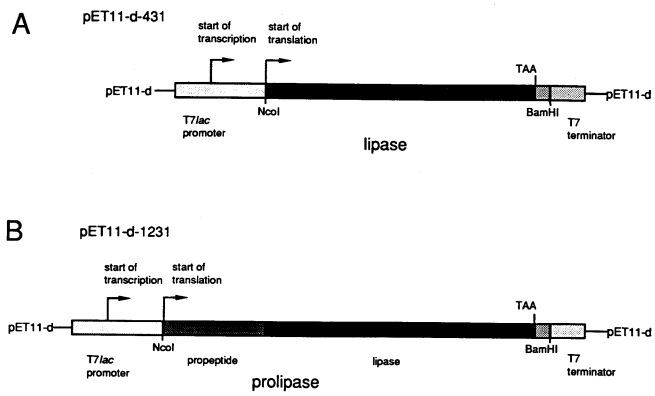

FIG. 2. Schematic representation of the lipase- and prolipase-encoding segments in the expression plasmid pET11-d (ref. 18). The lipase and prolipase genes were cloned as *Nco*I-*Bam*HI fragments between the T7*lac* promoter and T7 terminator sites in plasmid pET11-d. The termination codon for lipase and prolipase is indicated by the nucleotide triplet TAA.

site (Fig. 1A). An already existing *Nco*I site within the coding region was removed using a primer 5′GAGTCCCACGGTGGC 3′ (Fig. 1B). This base change did not alter the predicted amino acid sequence, but replaced the histidine codon, CAU, with the histidine codon, CAC, found considerably more frequently in mRNA from highly expressed genes of *E. coli* (17). Both primers were employed in a single mutagenesis experiment. In a second mutagenesis, a primer, 5′ACAGCGCCATGGATGGTGG 3′, was used to replace the codon for the *N*-terminal amino acid serine of the mature lipase enzyme (12) with the codon representing methionine (see Fig. 1C). This change also introduced an *Nco*I site. Phage carrying mutagenized cDNA inserts were identified by restriction enzyme cleavage of their double-stranded DNA. *Nco*I-*Bam*HI fragments, approximately 800 base pairs (bp) and 1100 bp in size, representing lipase- and prolipase-encoding DNA respectively, were isolated and ligated to vector pET11-d (18) that had been cleaved with *Nco*I and *Bam*HI (Fig. 2). The resulting plasmids, pET11-d-431 (Fig. 2A) and pET11-d-1231 (Fig. 2B), were introduced into *E. coli* BL21 (DE3) (18) by transformation.

Induction of lipase gene expression. E. coli BL21 (DE3) harboring the pET11-d recombinant plasmids were grown on solid LB agar medium (19) containing ampicillin (100 μg/mL). Cells from a single colony were inoculated into LB broth (19) containing ampicillin (100 μg/mL) and grown overnight at 30°C with light shaking. The culture was then diluted 100-fold into medium made up of the components of LB and of M9 minimal medium (19), pH 7.4, containing ampicillin (200 μg/mL). The cultures (500 mL of medium in 2 L baffled flasks) were shaken vigorously at 37°C until the optical density at 600 nm reached a value between 0.6 and 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM. Incubation was resumed, and the cells were harvested by centrifugation 3 to 4 h after the addition of IPTG. For experiments using lactose as inducer, the sugar was added to a final concentration of 10 mM.

Refolding of lipase. The cells obtained from a 1-L culture were resuspended in 50 mL of 50 mM Tris-HCl, pH 8, 5 mM ethylenediaminetetraacetic acid (EDTA), 10% sucrose. Lysozyme was added to a final concentration of 0.8 mg per mL. The mixture was incubated for 30 min at room temperature. Four-hundred mL of Triton buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5% Triton X-100) were added. After mixing, the lysate was sonicated to reduce viscosity and centrifuged at 15,000 × *g* for 30 min at 4°C. The insoluble pellet was resuspended in 400 mL of the Triton buffer and sonicated briefly. The resulting suspension was centrifuged at 15,000 × *g* for 20 min. The pellet was resuspended in 400 mL of 10 mM Tris-HCl, pH 8, 1 mM EDTA, and the suspension was centrifuged as above. The pellet was finally washed with distilled water and resuspended in 10 mL of distilled water. This suspension was added to 200 to 400 mL of 8 M urea [previously deionized by passage through mixed-bed resin AG 501-X8 (Bio-Rad Laboratories, Hercules, CA)] in 25 mM sodium phosphate, pH 7, 1 mM EDTA, 5 mM dithiothreitol (DTT). The solution was incubated at room temperature for 1 h, then centrifuged at 15,000 × *g* for 30 min at room temperature. Cystine (dissolved in 0.6 N NaOH) was added to a final concentration of 15 mM. The pH of the solution was maintained at 8.5 to 9 for 10 min. Then the solution was slowly added to 10 to 20 vol of cold (4–10°C) 50 mM sodium phoshate, pH 7.5–8, 1 mM EDTA, 5 mM cysteine. This solution was incubated for at least 48 h at 4°C to allow refolding of the denatured protein.

Purification of lipase and prolipase. The pH of the refolding solution was adjusted to 6.5 for lipase and 6.0 for prolipase by the dropwise addition of H_3PO_4. The solu-

tions were filtered through Whatman No. 1 paper (Whatman International Ltd, Maidstone, England) and concentrated in a stirred ultrafiltration cell using Diaflo ultrafiltration membranes YM10 or YM30 (Amicon, Beverly, MA) or by filtration through Minitan ultrafiltration filterplates type PLGC (Millipore, Bedford, MA).

The concentrated solution containing refolded lipase was filtered through Whatman No. 1 filter paper and loaded onto an oleic acid (OA) affinity chromatography column (16 × 250 mm) prepared as described previously (8). The column was washed with 200 mL 20 mM sodium phosphate, pH 6.5, 5% NaCl, followed by 200 mL 20 mM sodium phosphate, pH 6.5. The lipase was eluted with a gradient of Triton X-100 (0–0.5%) in 20 mM sodium phosphate, pH 6.5. For applications where the presence of detergent was not desirable, 60% ethyleneglycol in 20 mM sodium phosphate buffer, pH 6.5 was used for elution. Fractions containing lipase activity in excess of 200 U/mL were pooled and loaded onto a 12 × 200 mm column of carboxymethyl-sepharose (CM-Sepharose) CCL-6B-100 (Sigma, St. Louis, MO) equilibrated with 20 mM sodium phosphate, pH 6.5. The column was washed with 40 mL of the same buffer, and the lipase was eluted with 200 mL of an NaCl gradient (0–500 mM) in 20 mM sodium phosphate, pH 6.5.

Refolded prolipase did not bind to the oleic acid affinity gel. The buffer of the concentrated refolding solution was therefore exchanged for 20 mM sodium phosphate, pH 6, by diafiltration, and the solution was loaded onto a CM-Sepharose column (12 × 200 mm) equilibrated with 20 mM sodium phosphate, pH 6. The prolipase was eluted with a 0–500 mM NaCl gradient in the same buffer.

General protein techniques. Sodium dodecylsulfate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (20). The molecular weight of proteins was estimated by comparison of their mobilities with those of protein standards (Dalton Mark VII-L, Sigma). Protein bands on gels were stained with Coomassie Brilliant Blue R. An estimate of the relative intensity of bands on dried Commassie Blue-stained gels was obtained by scanning with a Bio-Rad Model 620 Video Densitometer (Bio-Rad, Richmond, CA) and analysis of the scanning profile with the Bio-Rad 1-D Analyst II programs.

Protein concentrations were determined by the method of Bradford (21) using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Bovine-γ-globulin was used as the standard.

N-Terminal amino acid sequences of recombinant lipase and prolipase were determined by automated Edman degradation using an Applied Biosystem (Foster City, CA) 473A Protein Sequencer. Determinations were made from 300 pmoles of protein dissolved in 0.1% trifluoroacetic acid.

Determination of lipase activity. Activity was determined qualitatively by spotting samples onto solid media containing olive oil (25 mL/L) emulsified in 20 mM sodium phosphate, pH 7.5, 0.001% Rhodamine B (Sigma). Lipase activity was visualized under ultraviolet light (22). Quantitative activity determinations were carried out titrametrically as described previously (8) using a VIT 90 Video titrator (Radiometer, Copenhagen, Denmark). The pH of the reaction mixture was maintained by the continuous addition of 0.1 N NaOH. For routine activity measurements, the assays were conducted at 25 °C at pH of 7.5 using purified olive oil (Sigma) as substrate. A thermostatted, water-jacketed reaction chamber was used for the determination of the effect of temperature on the lipase activity. Lipase activity was calculated from the maximum rate of titrant addition using a lipase titrametric assay program (Cichowicz, D.J. *et al.*, unpublished). A unit (U) of activity released one μmole of fatty acid per minute.

RESULTS AND DISCUSSION

Expression of recombinant lipase and prolipase. Over the years, *E. coli* has proven to be a suitable and often preferable organism for the overexpression of both pro- and eukaryotic genes. The ability of this bacterium to synthesize lipolytic protein from *R. delemar* cDNA cloned in lambda gt11 was crucial in the original isolation of the lipase cDNA (12). This cDNA was subsequently cloned in plasmid pUC8-2 (23), and, after addition of IPTG, *E. coli* cells harboring the resulting recombinant plasmid pUC8.2-14 (12) produced lipolytic protein with the same positional specificity as that of lipase produced by the fungus (12). However, only small amounts of lipolytic protein were present in cell-free extracts from induced recombinant *E. coli*, and the protein species that cross-reacted with polyclonal antibody raised against the fungal lipase were all larger than the 30 kDa molecular mass of mature *R. delemar* lipase (8,12). This indicated that *E. coli* JM101 was unable to fully process precursor proteins. Therefore, to obtain recombinant lipase corresponding to the mature fungal enzyme, it was necessary to remove the portions of the *R. delemar* lipase cDNA that encode the pre- and propeptide fragments (Fig. 1C). A DNA fragment encoding prolipase was also generated by removal of the part of the cDNA encoding the fungal signal peptide (Fig. 1A). The availability of a prolipase clone was desirable, because it was unknown if the propeptide was required for or beneficial to obtaining active lipase.

Many factors are known to determine the ultimate level of expression of a cloned gene. Among those factors are transcription rate and stability of the mRNA, rate of translation and stability of the protein product and also the amount of protein that can be accumulated before toxic effects occur. Attempts to clone the *R. delemar* lipase cDNA, excised from pUC8-2.14, into expression vectors pRX-1 (24) allowing transcription from a *trp* promoter or into the pMAL vectors (New England Biolabs, Beverly, MA) allowing the production of MaLE fusion proteins after transcription from a *lac* promoter, demonstrated that the presence of the cDNA in the correct orientation with respect to the promoters lead to plasmid instability and impaired viability even in the absence of inducer substances (data not shown). Thus the protein species produced from the cDNA have toxic properties which could lead to the selection of recombinant strains with low expression levels. The toxicity might be caused by the presence of the fungal signal peptide in the recombinant polypeptides or by lipolytic activity of the protein species produced from the cDNA. As mentioned above, the mutagenesis outlined in Figure 1 allowed the isolation of lipase- and prolipase-encoding DNAs that lack the sequences coding for the fungal signal peptide. To further improve the chances for successful expression of these DNAs in

E. coli, an expression system was sought that would provide a tighter control over transcription than either *trp* or *lac* promoters could provide. Such a system should prevent the appearance of damaging levels of lipolytic recombinant protein during the growth phase of the cells in the absence of an inducing substance.

Studier *et al.* (18) developed an *E. coli* expression system that included an additional layer of gene control. The vector components (pET plasmid series) place the transcription of insert DNA under the control of a bacteriophage T7 gene 10 promoter. This promoter is recognized by T7 RNA polymerase, which is in turn regulated by a *lac* promoter in the host component of this expression system, *E. coli* BL21 (DES3) (18). The activity of low levels of T7 RNA polymerase synthesized under noninducing conditions is inhibited by the presence of intracellular lysozyme produced in strain *E. coli* BL21 (DE3)[pLysS] (18). Using a derivative of this system, Deng *et al.* (25) were able to overexpress bovine pancreatic phospholipase A_2, an enzyme toxic to *E. coli*. The phospholipase accumulated in the periplasm of *E. coli* since the phospholipase gene had been fused to DNA encoding the OmpA signal peptide from *E. coli*. We attempted to express the *R. delemar* lipase and prolipase genes using the system employed by Deng *et al.* (25). However, recombinant lipase and, to a lesser extent, recombinant prolipase were still toxic to *E. coli* BL21 (DE3)[pLysS].

The toxic effect of the expressed lipase, which manifested itself even under noninducing conditions, necessitated a more tightly controlled expression system for recombinant *R. delemar* lipase. Therefore plasmid pET11-d (18) was selected. It is similar to the vector of the expression system described above, but it contains, as additional control features, a *lac* operator site (required for *lac* repressor binding) located in the T7 promoter region and also the gene encoding the *lac* repressor. The lipase and prolipase genes, on their respective *Nco*I-*Eco*RI fragments, were cloned into one of these plasmids, vector pET11-d (18) (Fig. 2). *E. coli* BL21 (DE3) cells containing the recombinant plasmids were stable under noninducing conditions. Upon induction with IPTG, the cells accumulated protein that migrated to positions on SDS-PAGE corresponding to the predicted molecular weights of prolipase or lipase (Figs. 3 and 4). Scanning of gels indicated that recombinant lipase constituted between 15 and 21% of the total cellular protein. Extracts from cells producing recombinant prolipase contained between 9 and 15% prolipase.

Lactose can be used as a substitute for the relatively expensive inducer substance IPTG. As was observed earlier with *E. coli* HB101 harboring a recombinant plasmid containing a *tac* promoter (26) and with *E. coli* BL21 harboring a recombinant pET3 plasmid (27), the appearance of recombinant protein was delayed by approximately one hour when lactose was added instead of IPTG. However, Neubauer *et al.*(27) demonstrated that lactose can be as efficient an inducer as IPTG provided that the addition of lactose was timed with respect to the glucose level in the growth medium. Therefore, lactose might be a suitable inducer for lipase-producing *E. coli* strains grown in fermentors equipped with glucose biosensors.

Lysates of cells containing the pET11-d derivatives and expressing the recombinant lipase or prolipase exhibited very little, if any, lipase activity (Tables 1 and 2). After low-

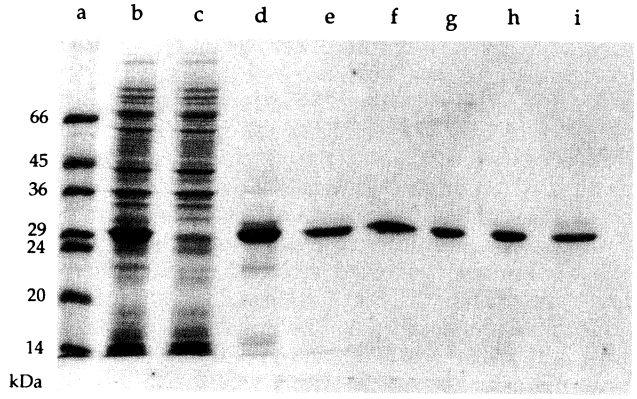

FIG. 3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of protein samples obtained during refolding and purification of recombinant *R. delemar* lipase. Lane a, molecular weight markers; lane b, lysate of *E. coli* BL21 (DE3) harboring pET11-d-431, induced for lipase expression; lane c, soluble fraction of lysate; lane d, buffer-insoluble fraction of lysate, dissolved in 8 M urea; lane e, concentrated refolding mixture to be subjected to affinity chromatography; lane f, combined fractions from affinity chromatography that exhibited significant lipase activity; lanes g and h, lipolytically active fractions eluted from CM-Sepharose column; lane i, *R. delemar* lipase purified from the fungal culture medium.

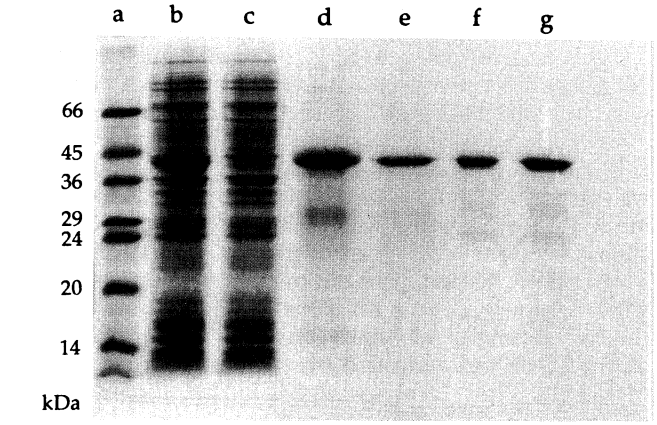

FIG. 4. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of protein samples obtained during refolding and partial purification of recombinant *R. delemar* prolipase. Lane a, molecular weight markers; lane b, lysate of *E. coli* BL21 (DE3) harboring pET11-d-1231, induced for prolipase expression; lane c, soluble fraction of lysate; lane d, buffer-insoluble fraction of lysate, dissolved in 8 M urea; lane e, concentrated refolding mixture, to be subjected to ion exchange chromatography on CM-Sepharose; lanes f and g, lipolytically active fractions eluted from CM-Sepharose.

speed centrifugation of cell lysates, the supernatant appeared to be devoid of the IPTG-inducible protein (Fig. 3, lane c), which was now found in the pellet. This is indicative of protein that has aggregated as inclusion bodies, a phenomenon often encountered with high expression systems (28). The highly expressed polypeptides remain in unfolded or partially folded states, and their sulfhydryl groups are mostly reduced (29). In this inactive state, lipase and prolipase polypeptides did not appear to cause cell lysis during the three- to four-hour induction period.

Refolding of recombinant lipase. The appearance of pro-

TABLE 1

Refolding and Purification of Recombinant *R. delemar* Lipase

Step	Protein[a] (mg)	Activity[b] (U)	Specific activity (U/mg)
Soluble fraction of lysate	1243	n.d.[c]	—
Insoluble fraction of lysate dissolved in 8 M urea	308	n.d.	—
Refolding solution (concentrated and filtered)	152	402234	2646
Combined fractions from oleic acid affinity column	104	397420	3821
Peak fractions CM-Sepharose column	62	315840	5094

[a]Bio-Rad protein assay.
[b]Assayed titrametrically. Units: μmole fatty acid released per min.
[c]None detected.

TABLE 2

Refolding and Purification of Recombinant *R. delemar* Prolipase

Step	Protein[a] (mg)	Activity[b] (U)	Specific activity (U/mg)
Soluble fraction of lysate	1246	n.d.[c]	—
Insoluble fraction of lysate dissolved in 8 M urea	170	n.d.	—
Refolding solution (concentrated and filtered)	117	429408	3670
Peak fractions CM-Sepharose column	59	373678	6333

[a]Bio-Rad protein assay.
[b]Assayed titrametrically. Units: μmole fatty acid released per min.
[c]None detected.

tein in insoluble form makes it necessary to devise a way to solubilize the polypeptides and to induce them to refold into an active form. The *R. delemar* lipase contains six cysteine residues which, based on amino acid sequence similarity with the lipase from *Rhizomucor miehei* (30,31), probably form three disulfide bridges. Therefore, refolding of recombinant *R. delemar* lipase has to include the formation of the proper disulfide bonds. The procedures necessary for successful refolding potentially add to the effort required for obtaining a pure protein, but there are advantages as well when protein is produced as inclusion bodies. First, a protein fraction highly enriched in the recombinant protein is obtained simply by separating the soluble from the insoluble fraction of a lysate. Second, recombinant protein is often synthesized in considerably higher amounts when deposited in inclusion bodies than when produced as soluble product. Thus, as shown for recombinant bovine interferon (32), the yield of active recombinant protein from cells producing inclusion bodies might be greater than that obtained from cells producing the recombinant protein in soluble form, even if only about 10% of the insoluble protein could be refolded properly.

The lipase-containing inclusion bodies were solubilized with 8 M urea, and unwanted inter- or intramolecular disulfide bonds that might have formed within the host cells or during isolation of the inclusion bodies were reduced with 5 mM DTT. In the resulting solution, 61 to 85% of the protein present co-migrated with purified *R. delemar* lipase on SDS-PAGE. Refolding was conducted on this solution of crude urea-solubilized inclusion bodies. The refolding procedure that gave the highest yield of refolded lipase was similar to that described for secretory leukocyte protease inhibitor (33). First, the urea-solubilized proteins were oxidized by the addition of cystine at a pH of 8.5 to 9. Then the urea concentration was reduced 10- to 20-fold by dilution to allow the folding process to commence. The dilution buffer contained cysteine to catalyze disulfide bond interchange. A pH of 7.5 to 8 in the dilution buffer gave the highest yield of active lipase.

During refolding, lipase displayed a tendency to aggregate and precipitate. Protein concentrations had to be kept below 100 μg/mL when diluting the urea-denatured protein in order to minimize this phenomenon. As was observed with other proteins (34,35), low temperatures during dilution also reduced the loss due to aggregation. The highest yield of active enzyme was achieved in refolding experiments conducted at 4 to 10°C. The yield was lower when the dilution buffer was at room temperature, and no active enzyme was obtained in experiments conducted at 37°C.

Refolding in buffer of pH 7.5 to 8 at 4°C was a relatively slow process and required several days for completion (Fig. 5). Some precipitate appeared during this time in the refolding solution. It was removed by filtration through Whatman No. 1 filter paper. In order to avoid strong precipitation during subsequent ultrafiltration and purification steps, the pH of the refolding solution was lowered to 6.5.

Purification of recombinant lipase. The mature recombinant lipase was purified by oleic acid affinity and ion

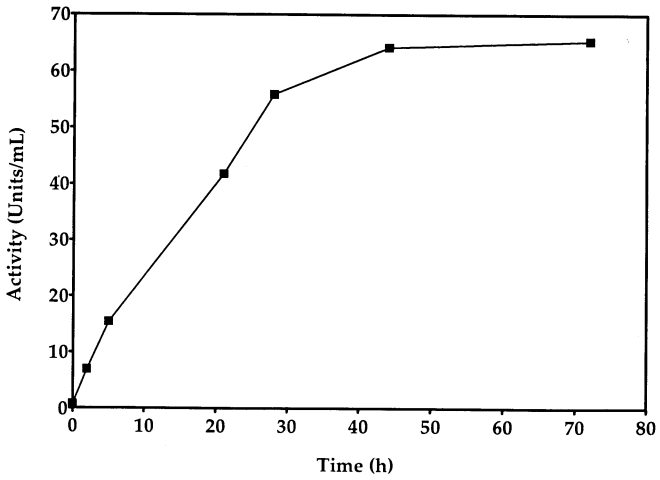

FIG. 5. Appearance of lipase activity after reduction of urea concentration from 8 M to 0.4 M. Samples were withdrawn from the refolding solution at the indicated time points and assayed for lipase activity as described in Materials and Methods.

exchange chromatography steps similar to those used for purification of the fungal enzyme (8). The activity recovered from 1 L of culture was almost completely retained by the 16 × 250 mm oleic acid affinity column. This activity was eluted virtually quantitatively as a single peak at a Triton X-100 concentration of approximately 0.25%. For purposes such as crystallization of the purified lipase, the presence of Triton X-100 might be undesirable due to its ability to bind to hydrophobic portions on proteins. Therefore an agent that presumably would not bind tightly to protein was sought to elute lipase from the OA affinity column. Ethylene glycol (60%) successfully eluted lipase from the column. The activity emerged in a broad peak, and trailing of activity was observed. Partially pure enzyme, recovered from the OA column either with Triton X-100 or ethylene glycol, was purified to apparent homogeneity by chromatography on CM-Sepharose at pH 6.5. (Fig. 3, lanes g–i). Migration of purified recombinant lipase on SDS-PAGE gels under reducing and nonreducing conditions was identical to that of lipase purified from *R. delemar*. The specific activity of fractions obtained after the ion exchange chromatography step was between 5000 and 8000 U per mg of protein. Similar specific activities were reported for purified lipase from *R. delemar* (8).

Amino acid sequence determination revealed that the *N*-terminal methionine was not removed post-translationally from the recombinant lipase. The second amino acid was predicted to be an aspartate (12); however, two sequencing runs failed to unambiguously discern between aspartate or asparagine as the identity of this residue. The sequence of the following 18 amino acids was identical to that determined for purified fungal-produced lipase and that predicted from the nucleotide sequence of the cDNA (12).

Refolding and partial purification of recombinant prolipase. Based on nucleotide sequence data (12), the *R. delemar* lipase was predicted to be synthesized as a proenzyme. The reason for the existence of the propeptide in lipase precursors is not known. Possibly, propeptides could be inhibitors of lipase, preventing membrane autolysis while the lipase is located inside the cell. This role for the propeptide was suggested for prophospholipase because the proenzyme did not exhibit interfacial activation and acted only on monomeric substrate (36). Thus prophospholipase might be unable to damage cellular phospholipid-containing structures.

It is also possible that the proregion is required for proper folding of the mature enzyme, as was observed for the *in vitro* and *in vivo* folding of some proteases (37,38). However, our data demonstrate that the propeptide is not required for *in vitro* refolding of mature recombinant *R. delemar* lipase. Whether or not the propeptide is involved in folding events *in vivo* or in some conformational changes during secretion from the fungal cell is unknown.

In vitro refolding of urea-denatured partially purified prolipase was conducted using the conditions used for the refolding of mature lipase. Yields from a refolding and purification experiment are shown in Table 2. The appearance of lipolytic activity measured titrametrically followed the same time course as that for lipase, and similar amounts of total activity were obtained from crude preparations of both proteins. Therefore the propeptide did not act as an inhibitor of recombinant lipase under the conditions employed. However, refolded recombinant prolipase behaved differently from recombinant mature lipase on solid media containing rhodamine B. Development of fluorescence was slower than was observed for the mature lipase, and fluorescent material was found in a diffuse zone around the site of sample application rather than only at the site of sample application. It is not known whether this was caused by physical differences, *e.g.*, mobility of the proteins in the agar medium, or by reactive differences that cause the differential removal of substrate or the generation of different products.

When purification was attempted in the same manner as for refolded recombinant lipase, recombinant prolipase did not bind to the oleic acid affinity column. This suggests that in the proenzyme the hydrophobic sites responsible for the binding of lipase to the affinity resin are obstructed or in some other manner unavailable for binding of the enzyme to the resin. Whether or not these differences between recombinant prolipase and lipase are important *in vivo* is unknown.

The refolded prolipase was purified only by ion exchange chromatography. The resulting preparations contained prolipase and a small amount of lower molecular weight proteins (Fig. 4, lanes f and g). The specific activities of such preparations ranged from 5000 to 8000 U/mg, comparable to those of purified fungal and recombinant mature lipase. The prolipase preparations were of sufficient purity to allow automated sequence determination of the first 20 amino acid residues. As was observed with recombinant lipase, the *N*-terminal methionine residue was still present. The sequence of the remaining 19 amino acid residues was as predicted from the nucleotide sequence of the cDNA (12).

Comparisons of physical properties of fungal lipase, recombinant lipase and recombinant prolipase. Haas *et al.* (8) have previously determined the influence of pH and temperature on the activity of purified fungal lipase. The lipase was most active at a pH around 8, near its predicted pI of 8.1, and the activity and stability were maximum at 30°C. The same characteristics were observed for the purified recombinant lipase (Fig. 6A–C). Recombinant prolipase was most active at pH 7.5, near its predicted pI

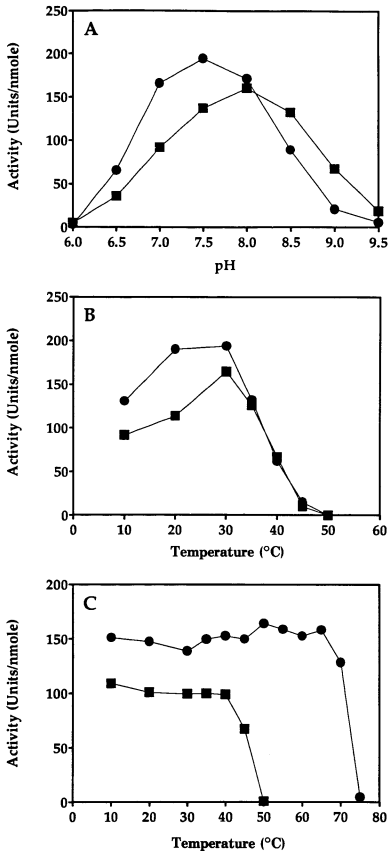

FIG. 6. A. Effect of pH on activity of lipase (—■—) and prolipase (—●—). Samples were assayed titrametrically as described in Materials and Methods. Activities were calculated based on the amount of protein used per assay and on molecular weights of 29,500 for lipase and 39,500 for prolipase. B. Effect of reaction temperature on activity of recombinant lipase and prolipase. Samples were assayed titrametrically in thermostatted reaction vessels as described in Materials and Methods. Activities were calculated as described for panel A. C. Thermal stability of lipase and prolipase. The enzyme samples were incubated at the indicated temperatures for 15 min and assayed at 25°C.

of 7.2 (Fig. 6A). The activity of prolipase was also maximum at 30°C (Fig. 6B). Thus, in their active conformation in the presence of substrate, both prolipase and lipase are equally susceptible to heat-induced denaturation. However, in the absence of substrate, prolipase, but not mature lipase, withstood exposure to temperatures as high as 70°C (Fig. 6C). Evidently, in the absence of an interface, the propeptide delays the onset of denaturation or promotes rapid renaturation of heat-exposed enzyme.

The similarities in the physical characteristics and the virtually identical specific activities suggest that the *R. delemar* lipase produced by *E. coli* and the lipase produced by the fungus could be of equivalent use for most applications. The yield of recombinant lipase after refolding and purification is about one-hundred times higher than that achieved by purification of the enzyme from fungal cultures. Thus a small culture provides sufficient enzyme material for most laboratory applications such as crystallography studies. It should be possible to increase the yield further simply by growing the recombinant *E. coli* in fermentors instead of the shaken flasks used in this study. Also, as our knowledge of protein refolding and appropriate refolding techniques advances, further yield increases should be possible.

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